(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012128 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/GB02/03372

(22) International Filing Date: 23 July 2002 (23.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0118786.3 1 August 2001 (01.08.2001) GB
0201161.7 18 January 2002 (18.01.2002) GB

(71) Applicant *(for all designated States except US)*: **PLANT BIOSCIENCE LIMITED** [GB/GB]; Norwich Research Park, Colney Lane, Norwich, Norfolk NR4 7UH (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BUTTNER, Mark, Jeremy** [GB/GB]; 44 Cambridge Street, Norwich, Norfolk NR2 3LD (GB). **HONG, Hee-Jeon** [KR/GB]; 2 Whitehall Road, Norwich, Norfolk NR2 3EW (GB). **PAGET, Mark, Sebastian, Boye** [GB/GB]; 11 Parkmore Terrace, Brighton, Sussex BN1 6AL (GB).

(74) Agents: **KREMER, Simon, M.** et al.; Mewburn Ellis, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012128 A2

(54) Title: DETECTION SYSTEM

(57) Abstract: Provided are methods and materials for detecting, in a sample, the activity of an antibiotic which affects cell integrity, which methods comprise the steps of: (a) providing a transformed Streptomyces coelicolor microorganism which comprises a nucleic acid encoding a promoter derived from a gene of the vanSRBFHAX cluster (e.g. from vanB = (SC66T3.03); vanF = (SC66T3.04); vanH (SC66T3.05), or vanR (SCH66.11c) of Streptomyces coelicolor operably linked to a heterologous reporter gene capable of causing a detectable signal, (b) contacting the sample with the transformed microorganism, (c) observing said transformed microorganism for said detectable signal. Also provided are processes for producing microorganisms for use in the method, and associated kits.

Detection System

Technical Field

The present invention relates to methods and materials for screening for compounds which have potential as antibiotics. It further relates to methods for generating microorganisms having utility in screening, tools which can be generally used in such methods, the microorganisms themselves, and biosensing methods employing the microorganisms.

Background Art

There is an ongoing requirement for novel compounds that have antibiotic activity, for instance to counteract the problem of drug resistance. Methods for screening potential sources of antibiotic that have been used in the prior art include those which are based on particular 'indicator' or 'reporter' strains of bacteria.

For instance, the use of *E. coli* PG8 (lacking both chromosomal β-lactamase and the cell wall biosynthetic enzyme PBP 1B) as an indicator strain led to the discovery of the monocyclic β-lactams [the nocardicins (Aoki *et al.*, 1976) and the monobactams (Imada *et al.*, 1981)].

Another screening approach has exploited the fact that the β-lactamase of *Bacillus licheniformis* is induced by β-lactams; the β-lactamase produced is easily detected by a chromogenic reaction, and this reporter system led to the independent discovery of the monobactams (Sykes *et al.*, 1981).

A *vanHp-cat* fusion (regulated by VanS/VanR - see Ulijasz *et al.* (1996) was used by Lai and Kirsch (1996) to assay more than 6,800 compound for induction of *vanHp* in *Enterococcus faecium*.

It can thus be seen that novel indicator systems would provide a contribution to the art.

Disclosure of the invention

The present inventors have provided a novel system to provide a broad-range, generic screen for antibiotics, and other compounds which target the cell envelope.

The system is based on the use of a promoter, linked to a suitable reporter gene, which promoter is regulated by a two-component signal transduction system. The two components are (i) a cell membrane located sensor kinase, and (ii) a trans-acting factor which is activated in response to a stimulus to the sensor such that it induces transcription of the reporter gene linked to the promoter.

As a test system, the inventors have established that a promoter from a gene of the vanSREFHAX cluster of *Streptomyces coelicolor* may be used as a generic detector of antibiotics or enzymes that interfere with the physical integrity of the cell envelope. This cluster contains four separate cistrons - *vanRS*, *vanE*, *vanF*, and *vanHAX* (see Figure 1). The designations and the equivalent gene designations in the *S. coelicolor* genome sequence (www.sanger.ac.uk/Projects/S_coelicolor/) are as follows: *vanR* = SCH66.11c; *vanS* = SCH66.10c; *vanE* = SC66T3.03; *vanF* = SC66T3.04; *vanH* = SC66T3.05; *vanA* = SC66T3.06; *vanX* = SC66T3.07.

Thus in a first aspect of the invention there is disclosed a method of detecting an activity of an antibiotic analyte in a sample comprising the steps of:

(a) providing a transformed microorganism which comprises a nucleic acid encoding a promoter derived from the vanSREFHAX cluster of *S. coelicolor* operably linked to a heterologous reporter gene capable of causing a detectable signal,

(b) contacting the sample with the transformed microorganism,

(c) observing said bacterium for said detectable signal;

The term "antibiotic" is used broadly in this aspect to include glycolipidic, glycopeptidic and peptidic compounds (natural, semi-synthetic or synthetic), which inhibit or kill (susceptible)

microorganisms generally by interfering with the physical integrity of the cell envelope. Suitable antibiotics include but are not limited to penicillins (such as amoxycillin, ampicillin, mezolocillin and ticarcillin), glycolipids (including phosphoglycolipidic compounds, for example moenomycin), glycopeptides (such as ristocetin and vancomycin) and peptides such as bacitracin and cephalosporins such as cephalosporin C, cefaclor, cephradine, and cephatrizine propylene glycol.

In preferred embodiments the invention is particularly effective for the detection of glycopeptide, peptide, and (phospho)glycolipidic compounds.

The activity detected may be correlated with the presence or absence of an antibiotic, or putative antibiotic, in the sample in a qualitative manner. Alternatively it may be used to make a quantitative assessment.

The promoter is preferably derived from *vanE* (or its homologues *vanF and H)*. In another embodiment the promoter is derived from *vanR*. These promoters are believed to be regulated by a two-component signal transduction system, which components are (i) a sensor kinase VanS, which will be receptive to changes in the cell envelope or membrane of the microorganism (and will generally be membrane bound) and (ii) a trans-acting factor VanR which is activated (e.g. phosphorylated) in response to stimulation of the sensor such that it activates the promoter and induces transcription of the reporter gene linked to the promoter.

The term "operably linked" refers to the linkage of a promoter to an RNA-encoding DNA sequence, and especially to the ability of the promoter to induce production of RNA transcripts corresponding to the DNA sequence when the promoter or regulatory sequence is recognized by a suitable polymerase. The term means that linked DNA sequences (e.g. promoter, reporter gene, terminator sequence) are operational or functional, i.e. work for their intended purposes.

By "observing" is meant ascertaining by any means (directly or indirectly) the presence or absence of the selected signal which is indicative of the binding event.

Thus the invention provides, *inter alia*, a method of detecting in a sample the activity of an antibiotic which affects cell integrity, which method comprises the steps of: (a) providing a transformed microorganism which comprises a nucleic acid encoding a promoter operably linked to a heterologous reporter gene capable of causing a detectable signal, (b) contacting the sample with the transformed microorganism, (c) observing said microorganism for said detectable signal, wherein the promoter is derived from a gene of the vanSREFHAX cluster of *Streptomyces coelicolor*.

Some particular embodiments and aspects will now be discussed in more detail.

Choice of sample

Samples may be selected from any suitable source. In particular, samples may be selected from culture supernatants and extracts from soil isolates, compounds produced by chemical synthesis including combinatorial chemistry; and compounds produced by combinatorial biosynthesis.

Also provided is a process of producing an isolated antibiotic which affects cell integrity, which method comprises the steps of: (a) performing a method as described above such as to identify the activity of the antibiotic in a sample, (b) isolating the antibiotic from the sample.

Choice of promoter

In preferred embodiments, the promoter is the *vanE* sequence or any of its homologues (*vanF and H*). In another embodiment the promoter is the *vanR* promoter. The nucleotide sequence of the *vanSREFHAX* cluster is published (www.sanger.ac.uk/Projects/S_coelicolor/).

Promoter sequences are also shown in Fig 1. The promoter may however be an active variant derived from these.

"Variants" in this context will have promoter activity, by which is meant the ability to bind an RNA polymerase (and other factors that initiate or modulate transcription under e.g. the VanR regulator) whereby an RNA transcript is produced from the reporter gene under the appropriate conditions i.e. activation of the component signal transduction pathway.

The term "derived" includes variants produced by modification of the authentic native sequence e.g. by introducing changes into the full-length or part-length sequence, for example substitutions, insertions, and/or deletions. This may be achieved by any appropriate technique, including restriction of the sequence with an endonuclease followed by the insertion of a selected base sequence (using linkers if required) and ligation. Also possible is PCR-mediated mutagenesis using mutant primers.

It may, for instance, be preferable to add in or remove restriction sites in order to facilitate further cloning. Modified sequences according to the present invention may have a sequence at least 70% identical to the sequence of the full or part-length inducible promoter or operon protein as appropriate. Typically there is 80% or more, 90% or more 95% or more or 98% or more identity between the modified sequence and the authentic sequence. There may be up to five, for example up to ten or up to twenty or more nucleotide deletions, insertions and/or substitutions made to the full-length or part length sequence provided functionality is not totally lost.

Similarity or identity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are preferably set, using the default matrix, as follows:

Gapopen (penalty for the first residue in a gap): -16 for DNA
Gapext (penalty for additional residues in a gap): -4 for DNA
KTUP word length: 6 for DNA.

Alternatively, homology in this context can be judged by probing under appropriate stringency conditions. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex.

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

Use of the promoters discussed above, particularly the *vanE* promoter or an active variant thereof, in the methods or systems described herein, forms one aspect of the present invention.

Choice of reporter gene

Generally the observed signal arises in consequence to an increased expression of a reporter protein from the reporter gene.

In the examples herein, the reporter protein provides resistance against a particular antibiotic (or otherwise lethal concentration of antibiotic). The detection in this case is the viability and/or increased rate of growth of the host microorganism in the presence of the antibiotic. This can be visualised e.g. directly, as a bacterial lawn on a nutrient plate. An example is the *neo* gene which confers resistance to both neomycin and kanamycin.

Alternative reporter genes may be used for increased ease of scoring and/or sensitivity. Most preferably the activity of the signal protein, or the protein itself, can be estimated photometrically (especially by fluorimetry or luminometry). This may be directly e.g. using instance green (and red) fluorescent protein, insect luciferase, and photobacterial luciferase. Alternatively it may be indirect e.g. whereby the signal gene causes a change which is detected by a colour indicator e.g. a pH change. In particular, the *lux* genes of *Vibrio harveyi* have been used successfully in *Streptomyces*.

Other suitable signal proteins (which have a readily detectable activity) are known in the art e.g. ß-galactosidase, which can generate a coloured substrate. The signal may utilise co-factors.

Methods for introducing signal genes into appropriate hosts are described in further detail below.

Integration of reporter gene

In one embodiment, the reporter gene may be introduced into the host such that it is operably linked to an appropriate existing inducible promoter. Typically this will be achieved by initiating targeted integration using aspects of the sequence forming part of the promoter region or operon.

Direct integration of a signal gene system such as luciferase (e.g. luxAB operon) into an environmentally responsive regulon in the host microorganism can be achieved by transposition or by illegitimate or legitimate recombination between a genetic construct introduced into the cell and the target operon or gene cluster located on either the chromosome or an episomal element.

In such cases, although the promoter is native to the cell, the reporter gene is "heterologous" to the promoter, by which is meant that the gene in question has been introduced into the cell using genetic engineering, i.e. by human intervention. Generally the heterologous gene will be non-naturally occurring in cells of that type.

Nucleic acid constructs

Preferably the nucleic acid encoding the promoter operably linked to the heterologous reporter gene capable of causing a detectable signal is in the form of an extrachromosomal vector.

"Vector", unless further specified, is defined to include, inter alia, any plasmid DNA, lysogenic phage DNA and/or transposon DNA, in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Strain derivatives encoding different gene dosage levels of the promoter/signal gene can be created by integration of the construct into the chromosome (low copy number/low sensitivity) or by use of medium or high copy number plasmids (medium or high sensitivity).

One example herein is based on the *neo* reporter plasmid, pIJ486, which was published in 1986 (Ward *et al.*, 1986) and has been used widely in the *Streptomyces* community to identify promoter-containing fragments and to quantify the strength of promoters *in vivo*. It has also been used to identify compounds which induce given promoters (e.g. Salah-Bey *et al.*, 1995; Murakami *et al.*, 1989). This has been used to generate the *vanEp-neo* fusion plasmid (pIJ6883) as described hereinafter.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression in common bacterial hosts. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic

acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Vectors for use in the invention will typically include: (a) a promoter which is regulated by a two-component signal transduction system operably linked to, (b) a heterologous reporter gene capable of causing a detectable signal, wherein the "two components" are (i) a sensor kinase (e.g. VanS), which will be receptive to changes in the cell envelope or membrane of the microorganism and (ii) a trans-acting factor (e.g. VanR) which is activated in response to stimulation of the sensor such that it activates the promoter.

In one assay described hereinafter, the *vanE* promoter is on a plasmid replicon with a copy number of 100-200 per chromosome. However the gene (*vanR*) encoding the activator of the promoter is present in single copy in the chromosome. In order to improve sensitivity it may be desired to increase the copy number of one or both of the genes encoding the two component system. For instance, in the assay employed in the examples below, additional copies of *vanR* may enhance the sensitivity of the assay. Similarly, changing the copy number of the sensor kinase gene (*vanS*) might have this effect.

One way of increasing copy number would be for the *vanRS* operon genes (optionally modified) to be included in a vector, optionally operably linked to a strong promoter or even the inducible promoter. The vector may or may not be the same vector as that carrying the inducible promoter and reporter gene.

Vectors as described above form one aspect of the present invention, as do methods of using them to produce a transformed host cell. The vector may remain discrete in the host. Alternatively it may integrate into the genome of the host.

Choice of host strain

The assay may use any suitable species of bacteria. Preferably the assay uses an actinomycete such as a strain of *Streptomyces* e.g. M600, which is a plasmid-free derivative of wild-type *S. coelicolor* A3(2). The *vanEpromoter-neo* fusion plasmid (pIJ6883) may then be introduced into the strain in question in order to perform the invention.

However it may be preferred to use other *Streptomyces* species that may have greater sensitivity to cell wall-specific antibiotics. For instance, specialised *S. coelicolor* host mutants may be employed that are defective in the cell wall and therefore hypersensitive to cell wall-specific antibiotics.

In addition, it may be preferred to use strains in which enzymes which may otherwise degrade antibiotics (thereby reducing the sensitivity of the assay) have been inactivated.

For example, it is known that *S. coelicolor* produces one or more β-lactamases (enzymes that inactivate β-lactams), and construction of a specialised host in which the β-lactamase structural genes have been inactivated would increase sensitivity of the bioassay in detecting β-lactams.

Similarly, disruption of the *vanHAX* operon might increase sensitivity of the bioassay in detecting vancomycin-related glycopeptides.

It may also be preferred to use genera of bacteria in which the promoter employed in the invention is non-native. This may permit different spectra of inducing compounds to be revealed. In such cases it may be required to express other heterologous genes in the bacteria in order to ensure the functionality of the two-component assay system. Generally the other genes may include transport and binding proteins, as well as the sensor and trans-acting factor(s).

As described above, the *VanRS* operon could be present on the same vector as that carrying the promoter and reporter gene, if preferred.

Examples of suitable bacteria may include, for example, the mycobacteria which are closely related to the streptomycetes, and include important human pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The *van* gene cluster (in single copy) and the *vanEp-neo* fusion (in multicopy) could be expressed in *Mycobacterium smegmatis* to permit screening therein.

Preferably, however, the host microorganism is the same species as that which provided the source of the inducible promoter, and will therefore naturally express the other components of the system required to give screening function.

The host cells described above, which may include vectors of the present invention, or heterologous reporter genes, form a further aspect of the present invention. An example transformed microorganism for use in a method described above would be one which is transformed with a vector of the present invention, optionally with a vector (which may be the same or different) encoding one or both of *VanRS* operon genes.

Systems

In a further aspect of the present invention there is provided a system for detecting an activity of an antibiotic in a sample comprising:

(a) a transformed microorganism as described above,
(b) means for detecting the signal produced from the reporter gene.

For example, where the signal is bacterial luciferase, this may be detected extracellularly using a photomultiplier or photodiode or any other photosensitive device.

Where the reporter gene encodes an antibiotic resistance gene (e.g. the *neo* gene in the Examples below) this could be used in parallel

processing system in which assays for multiple potential inducers are assessed using multiple nutrient plates. Alternatively, using an automated 96-well microtitre dish assay, kanamycin-resistant growth may be assayed automatically by optical density.

Naturally the methods and systems of the invention described above could be used as a primary screen, with further screens (e.g. based on antibiosis of target organisms, which may be different species to the screening microorganism) being employed to further exclude compounds not having the desired activity.

Kits

Also embraced within the scope of the present invention are kits for performing the various aspects of the invention. For instance a kit suitable for use in the first aspect may comprise a preparation of the microorganism, plus further means for carrying out the contact or observation steps e.g. buffers, co-factors (e.g. luciferin for addition to luciferase).

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments falling within the scope of the present invention will occur to those skilled in the art in the light of these.

Figures and Tables

Figure 1(a) shows a schematic diagram of how an antibiotic activates various promoters of the vanSREFHAX cluster. Figure 1(b) shows the cluster and relevant promoter regions.

Figure 2 shows the construction of pIJ6883. The second part of the Figure shows the conditions used.

Figure 3 shows the induction by vancomycin of the *vanR, vanE, vanF* and *vanH* promoters.

Table 1 summarises the results of the experiment described in Example 2, in which 13 antibiotics were tested for ability to induce the *vanE* promoter.

Examples

Example 1 - construction of *vanEp* reporter construct

A ~270 bp DNA fragment carrying the *vanE* promoter (*vanEp*) (SC66T3.03) was generated by the Polymerase Chain Reaction (PCR) using two synthetic oligonucleotide primers (vanE1; 5'-CCCCAAGCTTACACTCAGCAGCTCCAACGCGGT-3': vanE2; 5'-CCCCGAATTCTGGTGGCGTTGGCAGCGCTGGTG-3') and genomic DNA of *S. coelicolor* M600 as a template. This PCR product was cloned into the vector pGEM®-T (Promega) to create pIJ6882, and the nucleotide sequence of the insert was confirmed using standard sequencing procedures to ensure no unintended changes had occurred.

The *vanEp* (SC66T3.03) fragment was re-isolated from pIJ6882 as a ~270 bp *EcoRI-HindIII* fragment (sites underlined in the oligonucleotide sequences) and cloned into the multicopy promoter probe plasmid pIJ486 (Ward *et al.*, 1986) cut with *EcoRI* and *HindIII*, such that expression of the vector aminoglycoside phosphotransferase gene (*neo*), which confers resistance to both neomycin and kanamycin, depends on *vanEp*. The resulting plasmid was designated pIJ6883 (see Fig. 2).

Example 2 - construction and testing of *vanEp* reporter strain

Plasmid pIJ6883 was introduced by protoplast transformation (Hopwood et al., 1985) into M600 (Chakraburtty and Bibb, 1997), a plasmid-free derivative of wild-type *S. coelicolor* A3(2), and was found not to confer resistance to kanamycin, even to 2μg ml^{-1} kanamycin (on MMT medium minus L - tyrosine; Katz *et al.*, 1983).

To see if the *vanE* (SC66T3.03) promoter could be induced by control-antibiotics known to target the cell envelope, spores of M600 carrying pIJ6883 were spread on MMT medium minus L-tyrosine

carrying a lethal concentration of kanamycin (5 or 10 μg ml^{-1}) and potential inducers were applied on paper discs to the freshly spread plates and left for 4 days at 30°C. The results are shown for eleven antibiotics shown in Table 1. Inducers of the *vanE* promoter (bacitracin, ristocetin and vancomycin) raised the level of expression of the *neo* gene and hence induced a halo of kanamycin-resistant growth around the disc. The glycopeptides ristocetin and vancomycin and the peptide bacitracin were shown to be potent inducers, as was the glycolipidic compound. In contrast, "negative control" antibiotics that target the ribosome (e.g. thiostrepton, streptomycin) or DNA gyrase (novobiocin) do not induce a halo.

Table 1 summarises the results of the assay for the antibiotics used. Thus, it is clear that the *vanE* promoter bioassay detects different antibiotics that have different targets associated with the cell envelope, thereby allowing the system to act as a screen for certain classes of antibiotic, such as a broad range of peptidic, glyolipidic and glycopeptidic antibiotics, and provides for a generic screen for cell envelope-specific antibiotics.

The sensitivity of the demonstrated reporter system is inducible and is sensitive to vancomycin at least down to a level of 300 ng. These results clearly show the utility of the system as a screen.

Example 3 - use of reporter strain in bioassay

In order to perform the assay of the invention, spores of the host microorganism , for example, M600 carrying pIJ6883, are spread on MMT medium carrying a lethal concentration of kanamycin (10 μg ml^{-1})at concentration of approximately 5 x 10^6 / 12cm^2 plate. Test compound is applied on paper discs to a number of freshly spread plates in parallel using a different concentration of the test compound in each disc. A halo of kanamycin-resistant growth indicates that the test compound represents a cell envelope-specific antibiotic.

Penicillins		Cephalosporins		Glycopeptides		Peptides		Glycolipids		'Negative control' antibiotics that do not target the cell wall	
Amoxycillin (25µg)	+	Cefaclor (30µg)	+	Vancomycin (30µg)	+	Bacitracin (5U)	+	Moenomycin A (30µg)	+	Novobiocin (Target - DNA gyrase) (50µg)	-
Ampicillin (25µg)	+	Cefatrizine propylene glycol (30µg)	+	Ristocetin (30µg)	+					Thiostrepton (Target - the ribosome) (50µg)	-
Mezolocillin (75µg)	+	Cephradine (30µg)	+	Chloroeremomycin (30µg)	+					Streptomycin (Target - the ribosome) (10µg)	-
Ticarcillin (75µg)	+	Cephalosporin C (100µg)	+								

Table 1. Eleven antibiotics known to target the cell envelope and three 'negative control' antibiotics that do not target the cell envelope were tested for their ability to induce the vanEp-neo fusion in the bioassay (+ = induced a halo of kanamycin-resistant growth; - = did not induce a halo of kanamycin-resistant growth).

Example 4 - construction of *vanRp* reporter construct

A ~210 bp DNA fragment carrying the *vanR* (SCH66.11c) promoter (*vanRp*) is generated by the Polymerase Chain Reaction (PCR) using two synthetic oligonucleotide primers (vanR1; 5'-CCCCAAGCTTCGCCCAGTGAACGCGGCAGCGTGTT-3': vanR2; 5'-CCCCGAATTCACACTCAGCAGCTCCAACGCGGTGT-3') and genomic DNA of *S. coelicolor* M600 as a template. This PCR product was cloned into the vector pGEM®-T (Promega) to create pVANR1, and the nucleotide sequence of the insert is confirmed using standard procedures to ensure no unintended changes have occurred.

The *vanRp* fragment is re-isolated from pVANR1 as a ~210 bp *EcoRI*-*HindIII* fragment (sites underlined in the oligonucleotide sequences) and is cloned into the multicopy promoter probe plasmid pIJ486 (Ward *et al.*, 1986) cut with *EcoRI* and *HindIII*, such that expression of the vector aminoglycoside phosphotransferase gene (*neo*), which confers resistance to both neomycin and kanamycin, depends on *vanRp*. The resulting plasmid is designated pVANR2.

Example 5 - construction of *vanFp* reporter construct

A ~200 bp DNA fragment carrying the *vanF* (ie SC66T3.04) promoter (*vanFp*) is generated by the Polymerase Chain Reaction (PCR) using two synthetic oligonucleotide primers (vanF1; 5'-CCCCAAGCTTCGACCCAGCCCGACCTGGCCCGGCCCA-3': vanF2; 5'-CCCCGAATTCCTGCCGGACGGTCGGCCGCGTCGGGG-3') and genomic DNA of *S. coelicolor* M600 as a template. This PCR product is cloned into the vector pGEM®-T (Promega) to create pVANF1, and the nucleotide sequence of the insert is confirmed using standard nucleotide sequencing procedures to ensure no unintended changes have occurred.

The *vanFp* fragment is re-isolated from pVANF1 as a ~200 bp *EcoRI-HindIII* fragment (sites underlined in the oligonucleotide sequences) and cloned into the multicopy promoter probe plasmid pIJ486 (Ward *et al.*, 1986) cut with *EcoRI* and *HindIII*, such that expression of the vector aminoglycoside phosphotransferase gene (*neo*), which confers resistance to both neomycin and kanamycin, depends on *vanFp*. The resulting plasmid is designated pVANF2.

Example 6 - construction of *vanHp* reporter construct

A ~200 bp DNA fragment carrying the *vanH* (ie SC66T3.05) promoter (*vanHp*) s generated by the Polymerase Chain Reaction (PCR) using two synthetic oligonucleotide primers (vanH1; 5'-CCCCAAGCTTGGACCTTCGACCTCTATATGAAGCGA-3': vanH2; 5'-CCCCGAATTCAACCGTGATTCTCGTTGCCGGGAGGA-3') and genomic DNA of *S. coelicolor* M600 as a template. This PCR product is cloned into the vector pGEM®-T (Promega) to create pVANH1, and the nucleotide sequence of the insert is confirmed using standard nucleotide sequencing procedures to ensure no unintended changes have occurred.

The *vanHp* fragment is re-isolated from pVANH1 as a ~200 bp *EcoRI-HindIII* fragment (sites underlined in the oligonucleotide sequences) and cloned into the multicopy promoter probe plasmid pIJ486 (Ward *et al.*, 1986) cut with *EcoRI* and *HindIII*, such that expression of the vector aminoglycoside phosphotransferase gene (*neo*), which confers resistance to both neomycin and kanamycin, depends on *vanHp*. The resulting plasmid is designated pVANH2.

References

Aoki *et al.* (1976) Nocardicin A, a new monocyclic β-lactam antibiotic. I. Discovery, isolation and characterisation. *J. Antibiotics*. 29: 492-500.

Chakraburtty, R. and Bibb, M.J. (1997) The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J Bacteriol* 179: 5854-5861.

Hopwood, D.A., Bibb, M..J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M.., Lydiate, D.J., Smith, C.P., Ward, J.M., and Schrempf, H. (1985) Genetic Manipulation of *Streptomyces*: A Laboratory Manual. Norwich: The John Innes Foundation.

Imada, A., Kitano, K., Kintana, K., Muroi, M., and Asai, M. (1981) Sulfazecin and isosulfazecin, novel β-lactam antibiotics of bacterial origin. *Nature* 289: 590-591.

Janssen, G.R., and Bibb, M.J. (1993) Derivatives of pUC18 that have *Bgl*II sites flanking a multiple cloning site and that retain ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* 124: 133-134.

Katz, E., Thompson, C.J., and Hopwood, D.A. (1983) Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *J Gen Microbiol* 129: 2703-2714.

Lai, M.H., and Kirsch, D.R. (1996) Induction signals for vancomycin resistance encoded by the *vanA* gene cluster in *Enterococcus faecium*. *J Bacteriol*. 40: 1645-1648.

Lonetto, M.A., Brown, K.L., Rudd, K.E., and Buttner, M.J. (1994) Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci USA* 91: 7573-7577.

Murakami, T., Holt, T.G., and Thompson, C.J. (1989) Thiostrepton-induced gene expression in *Streptomyces lividans*. *J Bacteriol* 171: 1459-1466.

Paget, M.S.B., Chamberlin, L. Atrih, A., Foster, S.J. and Buttner, M.J. (1999a) Evidence that the ECF sigma factor, σ^E, is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J Bacteriol* 181: 204-211.

Paget, M.S.B., Leibovitz, E., and Buttner, M.J. (1999b) A putative two-component signal transduction system regulates σ^E, a sigma

factor required for normal cell wall integrity in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 33: 97-107.

Salah-Bey, K., Blanc, V., and Thompson, C.J. (1995) Stress-activated expression of a *Streptomyces pristinaespiralis* multidrug resistance gene (*ptr*) in various *Streptomyces* spp. and *Escherichia coli*. *Mol. Microbiol.* 17: 1001-1012.

Sykes, R.B *et al.* (1981) Monocyclic β-lactam antibiotics produced by bacteria. *Nature* 291: 489-491.

Sugiura, A., Hirokawa, K., Nakashima, K., and Mizuno, T. (1994) Signal-sensing mechanisms of the putative osmosensor KdpD in *Escherichia coli*. *Mol Microbiol* 14: 929-938.

Ulijasz, A.T., Grenader, A., and Weisblum, B. (1996) A vancomycin-inducible *lacZ* reporter system in *Bacillus subtilis*: induction by antibiotics that inhibit cell wall synthesis and by lysozyme. *J Bacteriol* 178: 6305-6309.

Walderhaug, M.O., Polarek, J.W., Voelkner, P., Daniel, J.M., Hesse, J.E., Altendorf, K., and Epstein, W. (1992) KdpD and KdpE, proteins that control expression of the *kdpABC* operon, are members of the two-component sensor-effector class of regulators. *J Bacteriol* 174: 2152-2159.

Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J., Buttner, M.J. and Bibb, M.J. (1986) Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. *Mol. Gen. Genet.* 203: 468-478.

Claims

1. A method of detecting in a sample the activity of an antibiotic which affects cell integrity, which method comprises the steps of:

(a) providing a transformed *Streptomyces coelicolor* microorganism which comprises a nucleic acid encoding

a promoter derived from a gene of the vanSREFHAX cluster of *Streptomyces coelicolor*

operably linked to a heterologous reporter gene capable of causing a detectable signal,

(b) contacting the sample with the transformed microorganism,

(c) observing said transformed microorganism for said detectable signal.

2. A method as claimed in claim 1 wherein the *Streptomyces coelicolor is S. coelicolor* A3(2).

3. A method as claimed in claim 1 wherein the *Streptomyces coelicolor is S. coelicolor* M600

4. A method as claimed in claim 1 wherein the promoter is derived from *vanE* = (SC66T3.03); *vanF* = (SC66T3.04) or *vanH* (SC66T3.05)

5. A method as claimed in claim 1 wherein the promoter is derived from *vanR* (SCH66.11c).

6. A method as claimed in any one of the preceding claims wherein the antibiotic is a glycolipidic, phosphoglycolipidic, glycopeptidic or peptidic compound which interferes with the physical integrity of the microorganism cell envelope or membrane.

7. A method as claimed in any one of the preceding claims wherein the reporter gene encodes a reporter protein which provides the microorganism with resistance to an antibiotic.

8. A method as claimed in claim 7 wherein the reporter gene is the *neo* reporter gene.

9. A method as claimed in any one of claims 1 to 6 wherein the reporter gene encodes a reporter protein which can be detected photometrically.

10. A method as claimed in any one of the preceding claims wherein the reporter gene is operably linked to the promoter by targeted integration into a sequence forming part of the promoter region or operon of the microorganism.

11. A method as claimed in any one of claims 1 to 9 wherein the nucleic acid encoding the promoter and heterologous reporter gene is a plasmid.

12. A method as claimed in claim 8 and claim 11 wherein the vector is derived from the *neo* reporter plasmid, pIJ486.

13. A method as claimed in claim 12 wherein the plasmid is pIJ6883 as described in Example 1 and Figure 2.

14. A method as claimed in any one of the preceding claims wherein the microorganism further comprises heterologous copies, or increased copy number, of (i) a gene encoding a trans-acting factor which activates the promoter, and\or (ii) a gene encoding a sensor kinase which is receptive to changes in the cell envelope or membrane of the microorganism and activates said trans-acting factor in response to the presence of antibiotics which affects cell integrity.

15. A method as claimed in claim 14 wherein the trans-acting factor is *vanR* (SCH66.11c) and the sensor kinase is *vanS* (SCH66.10c).

16. A method as claimed in any one of the preceding claims wherein the sample is selected from: a culture supernatant; a soil isolate; the product of combinatorial chemical synthesis; the product of combinatorial biosynthesis.

17. A method as claimed in any one of the preceding claims wherein the activity is qualitatively correlated with the presence

or absence of an antibiotic.

18. A method as claimed in any one of the preceding claims wherein the activity of the sample is further screened for antibiosis of a target organism.

19. A process of producing a transformed *Streptomyces coelicolor* microorganism for use in a method of any one of the preceding claims, which process comprises transforming a *Streptomyces coelicolor* microorganism either:
(i) by causing targeted integration, such that it is operably linked to a promoter of a gene of the vanSREFHAX cluster, of a heterologous reporter gene capable of causing a detectable signal, or
(ii) with a heterologous vector comprising promoter derived from a gene of the vanSREFHAX cluster of *Streptomyces coelicolor,* operably linked to a heterologous reporter gene capable of causing a detectable signal.

20. A process as claimed in claim 19 wherein the promoter is the *vanE* = (SC66T3.03); *vanF* = (SC66T3.04), *vanH* (SC66T3.05) or *vanR* (SCH66.11c) promoter.

21. A process as claimed in claim 19 or claim 20 wherein the microorganism is further transformed with heterologous copies of (i) a gene encoding a trans-acting factor which activates the promoter, and\or (ii) a gene encoding a sensor kinase which is receptive to changes in the cell envelope or membrane of the microorganism and activates said trans-acting factor in response to the presence of antibiotics which affects cell integrity.

22. A process of producing an isolated antibiotic which affects cell integrity, which method comprises the steps of:
(a) performing a method according to any one of claims 1 to 18 such as to identify the activity of the antibiotic in a sample,
(b) isolating the antibiotic from the sample.

23. A process as claimed in claim 22 which is preceded by the step of providing a transformed microorganism according to the

process of any one of claims 19 to 21.

24. A transformed microorganism for use in a method of any one of claims 1 to 18, which microorganism is a transformed *Streptomyces coelicolor* microorganism which either:
(i) comprises a heterologous reporter gene capable of causing a detectable signal, which reported gene is operably linked to a promoter of a gene of the vanSREFHAX cluster, or
(ii) is transformed with a heterologous vector comprising promoter derived from a gene of the vanSREFHAX cluster of *Streptomyces coelicolor*, operably linked to a heterologous reporter gene capable of causing a detectable signal.

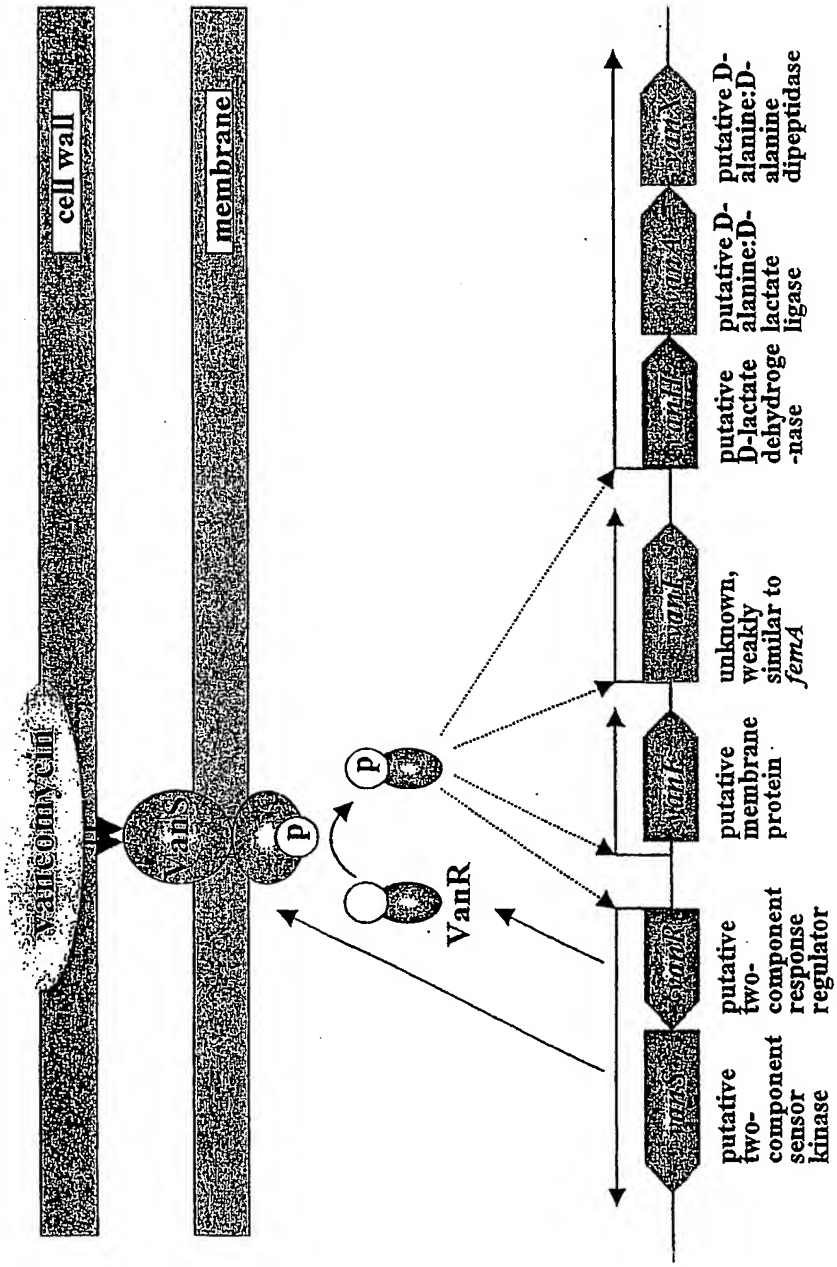
25. A transformed microorganism as claimed in claim 24 wherein the microorganism further comprises heterologous copies or increased copy number of (i) a gene encoding a trans-acting factor which activates the promoter, and\or (ii) a gene encoding a sensor kinase which is receptive to changes in the cell envelope or membrane of the microorganism and activates said trans-acting factor in response to the presence of antibiotics which affects cell integrity.

26. A system for detecting an activity of an antibiotic in a sample comprising:
(a) the transformed microorganism of claim 24 or claim 25,
(b) means for detecting the signal produced from the reporter gene.

27. A system as claimed in claim 26 wherein the means are a photosensitive device.

28. A system as claimed in claim 26 or claim 27 which is a parallel processing system in which detection of multiple activities is assessed using multiple cultures of transformed microorganisms.

29. A kit for performing a method according to any one of claims 1 to 18, which kit comprises a preparation of the microorganism of claim 24 or claim 25, plus further means for carrying out the contact or observation steps.



Model for the regulation of the *vanE*, *vanF* and *vanH* promoters in response to vancomycin

Figure 1a

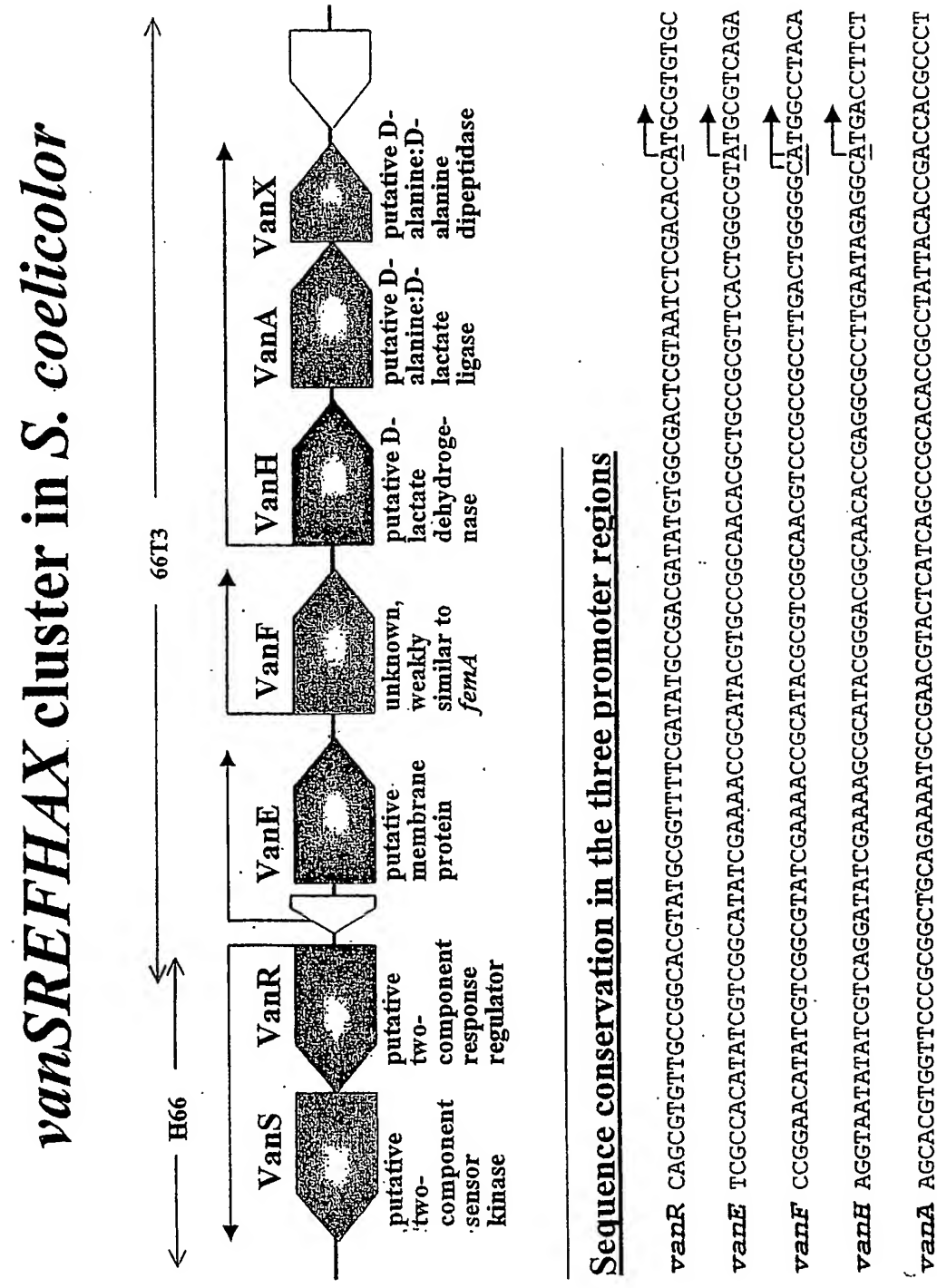

vanSREFHAX cluster in S. coelicolor
H66
66T3
VanS
VanR
VanE
VanF
VanH
VanA
VanX
putative two-component sensor kinase
putative two-component response regulator
putative membrane protein
unknown, weakly similar to femA
putative D-lactate dehydrogenase
putative D-alanine:D-lactate ligase
putative D-alanine:D-alanine dipeptidase
Sequence conservation in the three promoter regions
vanR CAGCGTGTTGCCGGCACGTATGCGGTTTTCGATATGCCGACGATATGTGGCGACTCGTAATCTCGACACCATGCGTGTGC
vanE TCGCCACATATCGTCGGCATATCGAAAACCGCATACGTGCCGGCAACACGCTGCCGCGTTCACTGGGCGTATGCGTCAGA
vanF CCGGAACATATCGTCGGCGTATCGAAAACCGCATACGCGTCGGCAACGTCCCGCCGCCTTGACTGGGGGCATGGCCTACA
vanH AGGTAATATATCGTCAGGATATCGAAAAGCGCATACGGGACGGCAACACCGAGGCGCCTTGAATAGAGGCATGACCTTCT
vanA AGCACGTGGTTCCCGCGGCTGCAGAAAATGCCGAACGTACTCATCAGCCCGCACACCGCCTATTACACCGACCACGCCCT
-35
-10

Figure 1b

Construction of pIJ6883 (pIJ486::*vanEp*)

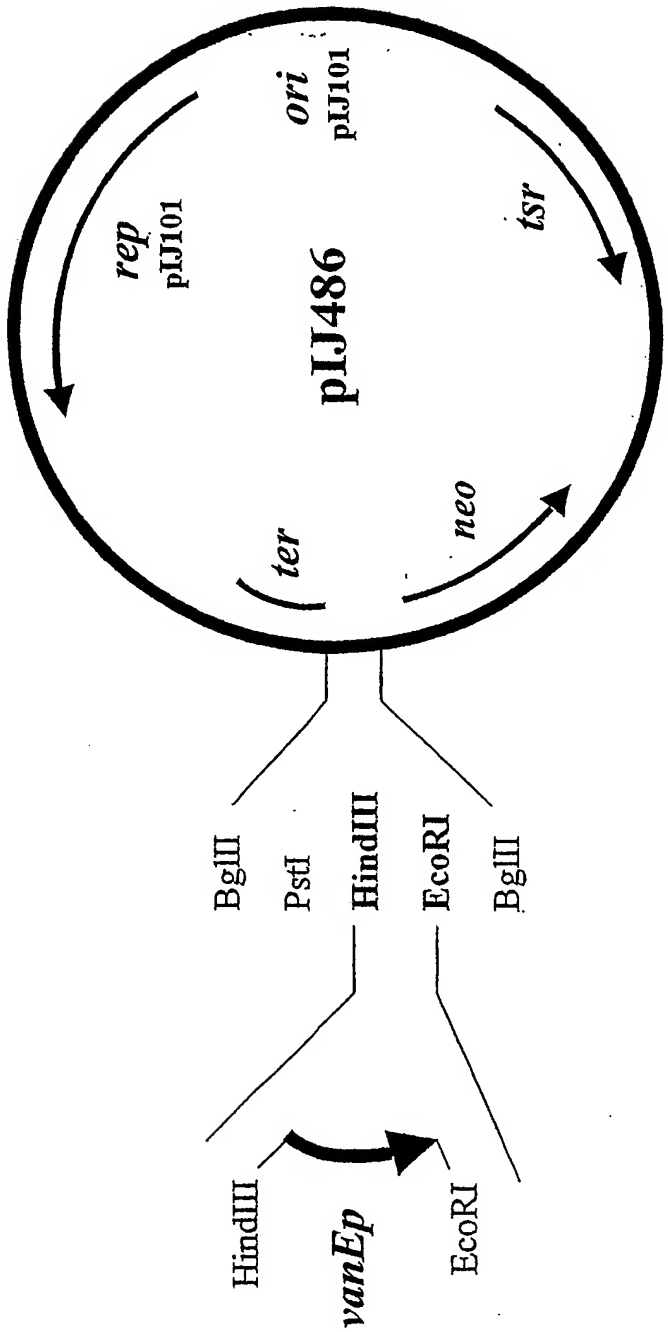


Figure 2

Ligation condition

Insert ----------16.5 µl
Plasmid ---------- 1.0 µl
T4 DNA ligase (Promega) -------- 0.5 µl
10X ligase butter ---------- 2.0 µl
} Total 20 µl

--> 16°C waterbath, overnight

PCR condition

10X PCR buffer ---------- 10.0 µl
dNTP (10 mM) ---------- 1.0 µl
oligo I (10 µM) ---------- 2.0 µl
oligo II (10 µM) ---------- 2.0 µl
glycerol 40% ---------- 12.5 µl
template DNA (M600 total DNA 200 ng/µl) ------- 1.0 µl
Taq DNA polymerase (Roche) ---------- 0.8 µl
dH_2O ---------- 54.5 µl
} Total 100 µl

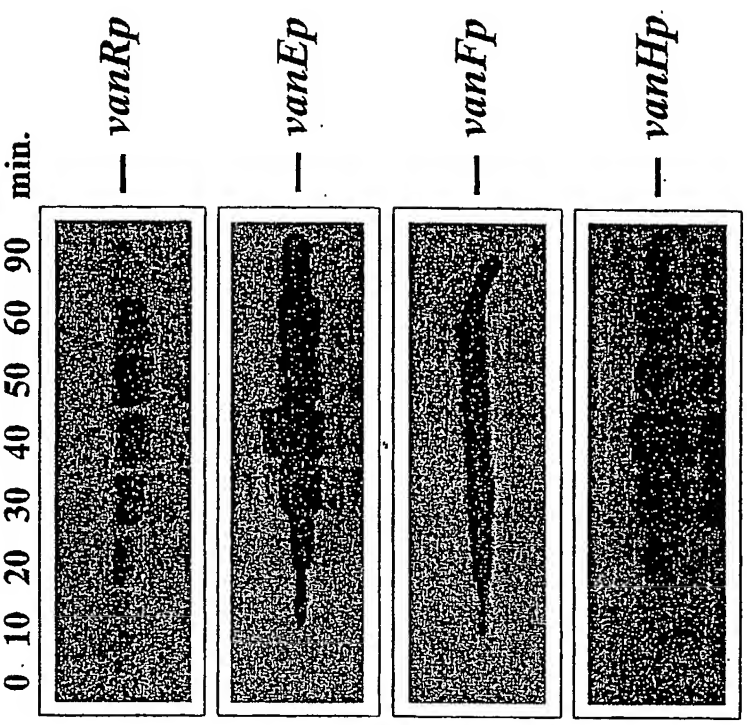
--> 95°C, 5 min.
--> 95°C, 50 sec.
55°C, 45 sec.
72°C, 45 sec.
} 28 cycle
--> 72 °C 5 min.

Purification condition for PCR fragment

QIAquick Gel Extraction Kit (QIAGEN)

Figure 2 Cont ...

The *vanR*, *vanE*, *vanF*, and *vanH* promoters are rapidly and strongly induced by vancomycin



S1 mapping of the *van* promoters using RNA isolated from M600 grown in NMMP liquid medium after induction with 10 µg/ml vancomycin

Figure 3